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<b>Applicant</b> BYASS, Louise, Jane et al	

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CLAIMS

1. Antifreeze polypeptides which can be obtained from carrots and which have an apparent molecular weight on SDS-PAGE of 36 kDa and isoforms or derivatives thereof.
2. Antifreeze polypeptides comprising one or more fragments (A-E) of the amino acid sequence as follows:
  - (A) LEU-PRO-ASN-LEU-PHE-GLY-LYS
  - (B) ILE-PRO-GLU-GLU-ILE-SER-ALA-LEU-LYS
  - (C) LEU-THR-X-LEU-ASP-LEU-SER-PHE-ASN-LYS
  - (D) SER-LEU-ARG-LEU-SER-SER-THR-SER-LEU-SER-GLY-PRO-VAL-PRO-LEU-PHE-PHE-PRO-GLN-LEU-X-LYS
  - (E) X-X-GLY-VAL-ILE-PRO-X-GLN-LEU-SER-THR-LEU-PRO-ASN-LEU-LYSand isoforms or derivatives thereof
3. Antifreeze polypeptides comprising the fragments (A-E) of claim 2.
4. Antifreeze polypeptides having an amino acid sequence as represented in Listing 1 and isoforms and derivatives thereof.

5. An isolated nucleic acid sequence encoding the antifreeze polypeptide of one or more of claims 1-4 and alleles thereof.
6. An isolated nucleic acid sequence substantially corresponding to the gene sequence of Listing I and alleles thereof.
7. Method of obtaining polypeptides according to one or more of claims 1-4 whereby the polypeptide is isolated from cold-acclimatised carrots.
8. Method of obtaining polypeptides according to one or more of claims 1-4, whereby the polypeptide is expressed by a genetically modified organism.
9. Method according to claim 8, whereby the organism is a microorganism, a plant or a cell culture.
10. An antibody capable of specifically binding the polypeptide of claim 1, 2, 3 or 4.
11. A polypeptide that is immunologically related to the polypeptide of claim 1, 2, 3 or 4 as determined by its cross reactivity with an antibody of claim 10.
12. Food product comprising a polypeptide of claim 1, 2, 3, 4 or 11 with the proviso that the food product is not a carrot containing the polypeptide at naturally occurring levels.

13. Food product of claim 12 being a frozen confectionery product or a frozen vegetable.
14. Method of producing a food product comprising an antifreeze polypeptide according to one or more claims 1, 2, 3 or 4, comprising the steps of
  - (a) adding to the food product a composition comprising said antifreeze polypeptide; or
  - (b) in situ production of said antifreeze polypeptide.
15. Use of the polypeptide of claims 1, 2, 3 or 4 for increasing the frost tolerance of plants.
16. Microorganisms, cell line or plant capable of expressing the polypeptide of claims 1, 2, 3 or 4, with the proviso that the plant is not an unmodified carrot plant.



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<b>(21) International Application Number:</b> PCT/EP97/06181 <b>(22) International Filing Date:</b> 6 November 1997 (06.11.97) <b>(30) Priority Data:</b> 96308362.1 19 November 1996 (19.11.96) EP <i>(34) Countries for which the regional or international application was filed:</i> NL et al. <b>(71) Applicant (for all designated States except AU BB CA GB GH IE KE LK LS MN MW NZ SD SG SZ TT UG US ZW):</b> UNILEVER N.V. [NL/NL]; Weena 455, NL-3013 AL Rotterdam (NL). <b>(71) Applicant (for AU BB CA GB GH IE KE LK LS MN MW NZ SD SG SZ TT UG ZW only):</b> UNILEVER PLC [GB/GB]; Unilever House, Blackfriars, London EC4P 4BQ (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> BYASS, Louise, Jane [GB/GB]; The University of York, Dept. of Biology, The Plant Laboratory, Heslington, York YO1 5YW (GB). DOUCET, Charlotte, Juliette [NL/GB]; The University of York, Dept. of Biology, The Plant Laboratory, Hes-		lington, York YO1 5YW (GB). FENN, Richard, Anthony [GB/GB]; Unilever Research Colworth Lab., Colworth House, Sharnbrook MK44 1LQ (GB). McARTHUR, Andrew, John [GB/GB]; Unilever Research Colworth Lab., Colworth House, Sharnbrook MK44 1LQ (GB). SIDEBOTTOM, Christopher, Michael [GB/GB]; Unilever Research Colworth Lab., Colworth House, Sharnbrook MK44 1LQ (GB). SMALLWOOD, Margaret, Felicia [GB/GB]; The University of York, Dept. of Biology, The Plant Laboratory, Heslington, York YO1 5YW (GB). WARRELL, Dawn [GB/GB]; The University of York, Dept. of Biology, The Plant Laboratory, Heslington, York YO1 5YW (GB). <b>(74) Common Representative:</b> UNILEVER N.V.; Patent Division, P.O. Box 137, NL-3130 AC Vlaardingen (NL). <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> CARROT ANTIFREEZE POLYPEPTIDES  <b>(57) Abstract</b> <p>Novel antifreeze polypeptides can be isolated from carrots. These peptides can favourably influence the properties of consumer products, e.g. frozen confectionary products.</p>		

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## CARROT ANTIFREEZE POLYPEPTIDES

Technical Field of the Invention

5 The invention relates to anti-freeze polypeptides (AFPs) and food product containing AFPs.

Background to the Invention

10 Anti-freeze polypeptides (AFPs) have been suggested for improving the freezing tolerance of foodstuffs.

For the purpose of the invention, the term AFP has the meaning as well-known in the art, namely those proteins which  
15 inhibit the growth of ice-crystals. See for example US 5,118,792.

WO 90/13571 discloses antifreeze peptides produced chemically or by recombinant DNA techniques from plants. The AFPs can  
20 suitably be used in food-products such as ice-cream. Example 3B shows modified ice-crystal shapes if a water-ice mixture is frozen into a film in combination with 0.01 wt% of AFP.

WO 92/22581 discloses AFPs from plants which can be used for  
25 controlling ice crystal shape in ice-cream. This document also describes a process for extracting a polypeptide composition from intercellular spaces of plants by infiltrating leaves with an extraction medium without rupturing the plant cells.

WO 94/03617 discloses the production of AFPs from yeast and their possible use in ice-cream. WO 96/11586 describes fish AFPs produced by microbes.

5 Up till now, however the use of AFPs has not been applied to commercially available consumer products. One reason for this are the high costs and complicated process for obtaining AFPs. Another problem is that sources of the AFPs are either difficult to obtain in sufficient quantities (e.g. fish  
10 containing AFPs) or are not directly suitable for use in food products.

The present invention aims to provide novel antifreeze polypeptides which have the advantage that they can easily be  
15 obtained from an abundant natural source and which provide good properties to products in which they are used.

It has been found that antifreeze polypeptides which possess good recrystallisation inhibition properties can be obtained  
20 from carrots. In particular it has been found that antifreeze polypeptides obtained from carrots show markedly better properties as compared to polypeptides isolated from other root vegetables. In particular the antifreeze polypeptides of the invention are capable of providing good recrystallisation  
25 inhibition properties without significantly changing the crystal shape of the ice-crystals, therewith possible leading to more favourable properties e.g. soft ice-cream.

Applicants have found that the effective antifreeze  
30 polypeptides from carrots are generally characterised by an apparent Molecular Weight on SDS-PAGE of 36 kDa. Accordingly in a first aspect the invention relates to antifreeze



polypeptides which can be obtained from carrots and which have an apparent molecular weight on SDS-PAGE of 36 kDa.

In this context it will be clear to the skilled person that due to variation e.g. in SDS PAGE, the apparent molecular weight can only be determined with some variation in the results. For the purpose of the invention these variations e.g. from 30 to 40 kDa or from 34 to 38 kDa are also embraced within the scope of the term "apparent Molecular Weight of 36 kDa".

Applicants also have found that the effective anti-freeze polypeptides according to the invention comprise fragments having an amino acid sequence as represented in the 15 examples.

Accordingly in a second aspect the invention relates to polypeptides comprising one or more fragments (A-E) having an amino acid sequence as follows:

20

(A) LEU-PRO-ASN-LEU-PHE-GLY-LYS

(B) ILE-PRO-GLU-GLU-ILE-SER-ALA-LEU-LYS

25 (C) LEU-THR-X-LEU-ASP-LEU-SER-PHE-ASN-LYS

(D) SER-LEU-ARG-LEU-SER-SER-THR-SER-LEU-SER-GLY-PRO-VAL-  
PRO-LEU-PHE-PHE-PRO-GLN-LEU-X-LYS

30 (E) X-X-GLU-VAL-ILE-PRO-X-GLN-LEU-SER-THR-LEU-PRO-ASN-LEU-  
LYS



5  
a  
493  
V I P P Q L S T L P N L K A L H L E R N -  
552

10  
a  
553  
E L T G E I P D I F G N F A G S P D I Y -  
612

15  
a  
613  
L S H N Q L T G F V P K T F A R A D P I -  
672

20  
a  
673  
R L D F S G N R L E G D I S F L F G P K -  
732

25  
a  
733  
K R L E M L D F S G N V L S F N F S R V -  
792

30  
a  
793  
Q E F P P S L T Y L D L N H N Q I S G S -  
852

35  
a  
853  
L S S E L A K L D L Q T F N V S D N N L -  
912

40  
a  
913  
C G K I P T G G N L Q R F D R T A Y L H -  
972

45  
a  
973  
N S C L C G A P L P E C \* -  
1011

### Listing 1

40

Also embraced within the invention are isoforms and derivatives of the above mentioned polypeptides which still possess the antifreeze properties. Preferable the derivatives show at least 75% homology with the polypeptide of Listing 1 or the polypeptide comprising the partial sequences (A-E),

more preferred more than 85%, most preferred more than 95%.  
For the purpose of the invention the term derivative also  
embraces modified polypeptides which still possess the  
antifreeze properties, for example glycosylated forms of the  
5 above polypeptides.

Also embraced within the invention are nucleotide sequences  
encoding the amino acids as described above. In particular  
the invention relates to nucleotide sequences of Listing 1  
10 and alleles thereof.

Also embraced within the invention are nucleotide fragments  
derived from the coding region that are capable of  
hybridizing to related genes that code for anti-freeze  
15 peptides.

Although the proteins of the invention can easily directly be  
isolated from carrots, also genetic manipulation techniques  
may be used to produce the proteins described in the  
20 invention.

An appropriate host cell or organism would be transformed by  
a gene construct that encodes the desired polypeptide. The  
nucleotide sequence coding for the polypeptide can be  
25 inserted into a suitable expression vector containing the  
necessary elements for transcription and translation and in a  
manner that they will be expressed under appropriate  
conditions (eg in proper orientation and correct reading  
frame and with appropriate targeting and expression  
30 sequences). The methods required to construct these  
expression vectors are well known to those skilled in the  
art.

A number of expression systems may be utilised to express the polypeptide coding sequence. These include, but are not limited to, bacteria, yeast, insect cell systems, plant cell  
5 culture systems and plants all transformed with the appropriate expression vectors. Yeast, plants and plant culture systems are preferred in this context.

A wide variety of plants and plant cell systems can be  
10 transformed with the nucleic acid constructs of the polypeptides. Preferred embodiments would include, but are not limited to, maize, tomato, tobacco, carrots, strawberries, rape seed and sugar beet.

15 One preferred embodiment of the invention relates to the use of AFPs of the invention for increasing the frost tolerance of plants. This case for example be done by the above method whereby the plants are transformed to ensure (increased) production of the AFPs of the invention, therewith increasing  
20 the frost tolerance of said plants.

The invention also relates to antibodies which specifically bind an (epitope of the) polypeptides of the invention. Also embraced are polypeptides which are immunologically related  
25 to the polypeptides as determined by its cross reactivity with an antibody raised against the above polypeptides.

Based on the above information it is also possible to genetically modify other natural sources such that  
30 they produce the advantageous AFPs as identified here-above.

Preferably those AFPs are chosen which have significant ice-recrystallisation inhibition properties. A suitable test for determining the recrystallisation inhibition properties is indicated in example I. Also preferably AFPs in accordance to  
5 the invention provide a ice particle size in the frozen product (mean crystal length) upon recrystallisation of less than 50  $\mu\text{M}$ , more preferred from 5 to 40  $\mu\text{M}$ .

The AFPs can conveniently be used in several products,  
10 preferably in food products which are frozen or intended to be frozen. Carrots which comprise the AFP at naturally occurring levels are not embraced within the scope of the invention. However, food product containing (parts) of carrots are embraced within this term. Also embraced are  
15 carrots which have been transformed to over express the AFP of the invention i.e. which contain the AFP at significantly higher levels than non-transformed carrots.

Examples of such food products are: frozen food products such  
20 as vegetables, sauces, soups, snacks, frozen confectionery products such as ice-cream or water-ice, dairy products etc.

The preferred products wherein the AFPs are used are or frozen vegetables or frozen confectionery products such as  
25 ice-cream or water-ice. Preferably the level of AFPs is from 0.00001 to 0.5 wt% based on the final product.

If dry-mixes or concentrates are used, the concentration may be higher in order to ensure that the level in the final  
30 frozen product is within the above ranges. Surprisingly it has been found that compositions of the invention can contain very low amounts of AFPs while still being of good quality.

Preferred levels of AFP are from 0.00001 to 0.5 wt%, more preferred 0.00005 to 0.3 wt%, most preferred 0.0001 to 0.2 wt%.

5

For the purpose of the invention it is not necessary to add the AFP in purified form to the food product. Also possible is to add a composition comprising AFPs e.g. an extract of the natural material which produces the AFP.

- 10 Also it is possible to modify the food product such that the AFP is produced in situ e.g. by adding genetically modified micro-organisms which are capable of producing the AFP in the food product, or even to genetically modify the food product (e.g. the vegetable) such that (the vegetable) in itself it  
15 is capable of producing the AFP in situ.

For the purpose of the invention the term frozen confectionery product includes milk containing frozen confections such as ice-cream, frozen yoghurt, sherbet,  
20 sorbet, ice milk and frozen custard, water-ices, granites and frozen fruit purees.

Preferably a the level of solids in the frozen confection (e.g. sugar, fat, flavouring etc) is more than 3 wt%, more  
25 preferred from 10 to 70wt, for example 40 to 70 wt%.

Frozen confectionery products according to the invention can be produced by any method suitable for the production of frozen confectionery. Especially preferably however all the  
30 ingredients of the formulation are fully mixed before the freezing process starts.

## EXAMPLES

### Example I

5 Carrots (*Daucus carota* cv Autumn King) were grown in individual pots. When plants were approximately twelve weeks old, they were transferred to a cold room and held at 4°C in constant light during 4 weeks for cold-acclimation. Plants were watered three times a week.

10

Fresh tissue of the carrots were ground with a pestle and mortar (cooled to 4°C) in an equal volume buffer A (10mM EDTA, 20 mM Ascorbic acid, buffered with Tris to pH 7.4) held on ice. The homogenates were filtered through one layer of 15 muslin and kept on ice prior to further use.

As a comparison several other root-plants were grown and homogenates prepared from the roots as above.

20 Anti-freeze activity was measured using a modified "splat assay" (Knight et al, 1988). 2.5 µl of the solution under investigation in 30% (w/w) sucrose was transferred onto a clean, appropriately labelled, 16 mm circular coverslip. A second coverslip was placed on top of the drop of solution 25 and the sandwich pressed together between finger and thumb. The sandwich was dropped into a bath of hexane held at -80°C in a box of dry ice. When all sandwiches had been prepared, sandwiches were transferred from the -80°C hexane bath to the viewing chamber containing hexane held at -6°C using forceps 30 pre-cooled in the dry ice. Upon transfer to -6°C, sandwiches could be seen to change from a transparent to an opaque



appearance. Images were recorded by video camera and grabbed into an image analysis system (LUCIA, Nikon) using a 20x objective. Images of each splat were recorded at time = 0 and again after 30-60 minutes. The size of the ice-crystals in both assays was compared. If the size at 30-60 minutes is similar or only moderately increased (say less than 20% increased, more preferred less than 10% increased, most preferred less than 5 % increased) compared to the size at t=0, this is an indication of good ice-crystal recrystallisation inhibition properties.

Results: from the sandwich splat assay test it appeared that samples from carrot roots, carrot stem and carrot leaves possess significant ice-recrystallisation inhibition properties, whereby the roots and leaves are most active. As a comparison a sample of non-acclimated carrot roots was tested, which showed significant less activity. For the following examples root tissue was used for further testing on carrots.

As a comparison several other vegetable roots were investigated by means of the sandwich splat assay test in 30% sucrose. Among these vegetables were turnip, kale, brussels sprout, wintergreen cabbage, rape, pak choi, parsnip and strawberry. None of these sources of material provided significant ice-recrystallisation inhibition activity.

Example II

Carrot root tissue was homogenized in three volumes (w/v) buffer (20mM ascorbic acid, 10 mM EDTA, 50 mM Tris/HCL, pH 7.2) in a pre-cooled pestle and mortar and filtered through one layer of muslin. The filtrate was centrifuged at 6,000 g, ten minutes at 4°C; the supernatant was collected and centrifuged at 100,000g for 1 hour at 4°C. The 100,000 g supernatant from this step is termed the soluble fraction and the pellet the microsomal fraction.

The supernatant was applied to a 30 ml fast flow Q Sepharose (Pharmacia) column pre-equilibrated in 50 mM Tris/HCL pH 7.4 at a flow rate of 5 ml/min supplied by a HiLoad pump P-50 controlled by a Gradifrac low pressure chromatography system (Pharmacia) at 4°C and the eluate monitored at OD 280 by a UV monitor (Monitor UV1, Pharmacia) recorded on a chart recorder (REC 102, Pharmacia). 5 ml fractions were collected. The column was washed with 50mM Tris/HCL pH 7.4 at the same flow rate until the OD 280 returned to zero. A 150ml gradient of 0-0.4 M NaCl in Tris/HCL pH 7.4 was then applied followed by a 2 M NaCl column wash. Eluate fractions were subjected to the splat assay as in example I.

Fractions containing anti-freeze activity as evidenced by recrystallisation inhibition were pooled and concentrated using polyethylene glycol as follows: the fractions were transferred in 10kDa cut off dialysis tubing (Sigma) which had been washed in tap water, boiled in 50mM EDTA pH 7.5 for 10 minutes and rinsed in milli Q water. The dialysis tubing containing the sample to be concentrated was covered with solid polyethylene glycol compound Mol. Wt. 15,000 - 20,000

(Sigma) and incubated at 4°C for up to 4 hours or until the sample volume inside the dialysis tubing had reduced up to 10 fold.

- 5 The pooled concentrate from the Q sepharose column was applied either to a phenyl Sepharose column, a SMART superdex 75 gel permeation column or an FPLC superdex 75 gel permeation column.
- 10 Carrot root anti-freeze proteins were purified by gel permeation chromatography as follows:
- 20µl aliquots of sample were applied to a SMART superdex 75 column (Pharmacia) pre-equilibrated in 50mM Tris/HCl pH7.4 containing 0.15M NaCl (Buffer E) at a flow rate of 40µl/min and components separated by gel permeation at the same flow rate in equilibration buffer. The eluate was monitored at OD 280 and OD 215. 80µl fractions were collected between 0.85 and 0.89ml, 40µl fractions between 0.89 and 1.24ml and 100µl fractions between 1.24 and 3.0 ml. The void volume (Vo) of the column was 0.91 ml as determined by the retention volume of a solution of Blue Dextran. The superdex column was calibrated by application of 10µl of a solution containing 5mg/ml BSA (Mr 66kDa, retention (Ve)=1.02 ml), 3mg/ml Carbonic anhydrase (Mr 29 kDa, Ve=1.22 ml), 2mg/ml Cytochrome C (Mr 12.4 kDa, Ve=1.41 ml) and 2mg/ml Aprotinin (Mr 6.5 kDa, Ve=1.59 ml) and a standard curve plotted of Ve/Vo against log Mr. Fractions containing anti-freeze activity were identified by the splat assays as described in Example I, with an activity peak that showed a retention volume of 1.16 ml and an apparent molecular weight of 40 kDa. These measurement

confirmed that the 36 kDa band from cold acclimatised carrots was an anti-freeze peptide.

SDS-PAGE was carried out according to Laemmli (1970) using  
5 the Biorad mini system. Samples to be analyzed by SDS-PAGE were dissolved in SDS-PAGE sample buffer (Laemmli 1970), heated for 5 minutes at 100°C on a dry heating block (Techne) and centrifuged for 3 minutes at 10,000g at room temperature. Samples (10-50µl) were applied to mini-gels (Biorad, 0.75, 1.0  
10 or 1.5mm thickness, 10, 12, 15% acrylamide or 10-20% gradient acrylamide (pre-poured from Biorad)) and electrophoretically separated. Separated polypeptides were fixed and stained in the gel either with Coomassie blue (0.1% (w/v) Coomassie Brilliant Blue in acetic acid/methanol/miliQ water (5:4:31,  
15 by vol)) or silver stained using the Biorad silver stain kit according to the manufacturer's instructions. Gels were dried between two sheets of Gelair cellophane in a Biorad gelair dryer according to the manufacturer's instructions. Sigma high and low range molecular weight marker kits were used  
20 according to the manufacturer's instructions for determination of apparent  $M_r$  on SDS-PAGE.

The ion exchange chromatography was carried out with cold acclimatised carrot root and non-cold acclimatised carrot  
25 root. The resulting gel SDS-PAGE gels showed the presence of a 36kDa band in the cold acclimatised sample. This band was much less abundant in the non-cold acclimatised root. This 36kDa band was hence attributed to anti-freeze activity.

Example III

For protein sequencing, the 36kDa carrot root protein was purified as described in the previous example and then to  
5 ensure further purification the sample to be sequenced was excised from the SDS PAGE gel and then proteolytically digested *in situ* in the polyacrylamide gel slice.

Preparations of largely pure 36 kDa protein, that still had  
10 some minor contaminating proteins, were loaded onto a 12% polyacrylamide gel. Three lanes each with 2 µg of protein were loaded and electrophoresed in the gel until the dye front reached the bottom of the gel. The gel was then stained in 0.2% uocmassie brilliant blue (w/v), 30% methanol (v/v),  
15 1% acetic acid (v/v) for 20 minutes and then destain with 30% methanol until the protein bands could be visualised. The 36 kDa band was identified by comparison with molecular weight markers loaded into adjacent lanes and the band from each lane was excised with a scalpel blade, taking care to exclude  
20 contaminating bands.

The gel slices were transferred to a clean eppendorf tube and washed twice with 0.5ml of 50% acetonitrile (v/v), 100mM Tris/Cl, pH 8.5. The washing removed some of the uocmassie  
25 stain and also partially dehydrated the gel slices. The gel slices were then removed from the tube and subjected to air drying on the laboratory bench until they had shrunk significantly and started to curl up. They were then transferred back to the eppendorf and rehydrated with  
30 firstly, 10µl of 100mM Tris/Cl, pH 8.5 containing 1µg of endoproteinase Lys C (Boehringer Mannheim). This is a proteinase that specifically cleaves polypeptide chains on

the carboxy terminal side of lysine residues. Further Tris buffer was added to the gel slices until they were fully rehydrated and they were then incubated at 37°C for 16 hours.

- 5 After incubation 1µl of trifluoroacetic acid was added to the tube to stop the reaction and then the gel slices were washed twice with 0.3ml of 60% acetonitrile (v/v), 0.1% TFA (v/v) at 30°C for 30 minutes. This was to again partially dehydrate the gel slices causing them to shrink and elute the peptides  
10 that had been generated. The supernatant was transferred to another clean eppendorf tube and then dried in a centrifugal evaporator for 2 hours until the sample was near dryness and resuspended to a volume of 0.1ml with 0.1% TFA.
- 15 The peptides were then separated by reversed phase HPLC on a Smart micropurification system (Pharmacia). The peptide digest was loaded onto a C18 column (2.1 x 100 mm) equilibrated in 0.1% TFA (Solvent A) at a flow rate of 0.1ml min. The column was then eluted with a gradient of 0 - 70% of  
20 Solvent B (90% acetonitrile v/v, 0.085% TFA v/v) over 70 minutes at the same flow rate. The optical density was monitored at 214 nm and individual peptide peaks were collected in the fraction collector by manual stepping. Polypeptides were sequenced by loading onto a model 492  
25 Perkin Elmer protein sequencer using the liquid phase chemistry cycles as recommended by the manufacturer.

Several polypeptide fragments (A-E) were analyzed in the 36 kDa band and had sequences substantially homologous to:

- 5
- (A) LEU-PRO-ASN-LEU-PHE-GLY-LYS
- (B) ILE-PRO-GLU-GLU-ILE-SER-ALA-LEU-LYS
- 10 (C) LEU-THR-X-LEU-ASP-LEU-SER-PHE-ASN-LYS
- (D) SER-LEU-ARG-LEU-SER-SER-THR-SER-LEU-SER-GLY-PRO-VAL-  
PRO-LEU-PHE-PHE-PRO-GLN-LEU-X-LYS
- 15 (E) X-X-GLY-VAL-ILE-PRO-X-GLN-LEU-SER-THR-LEU-PRO-ASN-LEU-  
LYS

Example IV-a carrot cell culture

A carrot cell suspension culture line (NOR) was obtained from the Department of Biochemistry and Molecular Biology,  
5 University of Leeds. The culture was maintained by subculturing 10 ml of the culture into 90 ml of fresh Murashige and Skoog medium (Sigma) containing 25 g/l sucrose and 1 mg/l 2,4-D every seven days. Cultures were incubated in an orbital shaking incubator at 150 rpm at 25°C in the dark.

10

The NOR culture was cold treated as follows:

NOR cultures were transferred to 4°C after 4d and 7d of growth at 25°C. Cultures were harvested at t=0, t=7d and  
15 t=14d. In addition to harvesting, the packed cell volume (PCV) was determined for each culture at each time point.

The media samples from NOR suspension cultures were analyzed as follows. Approximately 1/10th of the volume of a frozen  
20 aliquot of conditioned suspension culture medium was allowed to defrost. The defrosted (freeze concentrated) portion was removed and tested for activity by sandwich splat assays as described in Example I. Medium from cold acclimated cultures was found to contain significantly more activity than medium  
25 from non-cold acclimated cultures.

The cold acclimated NOR carrot medium was buffered by addition of 100µl of 1M Tris/HCl pH 7.4. Purification of activity was then performed by ion exchange and gel  
30 permeation chromatography using a method based on that in Example II: the buffered medium was applied to a 1 ml Q Sepharose column (Pharmacia) at a flow rate of 1 ml/min and



bound molecules eluted with 3 ml aliquots of 500 mM Tris/HCl pH 7.4 containing concentrations of NaCl starting at 0.1 M and increasing to 0.5 M in 0.1 M steps. 1 ml fractions were collected and tested for activity as in Example I.

5

The antifreeze activity in the active ion exchange fractions was purified by gel permeation chromatography as follows. The active fraction from above was acetone precipitated and the pellet resuspended in 50 $\mu$ l 50mM Tris/HCl + 0.15 M NaCl, pH 10 7.2. This was then centrifuged at 10.000 g for 10 minutes, and 20 $\mu$ l loaded onto a Superdex 75 gel permeation column on the Pharmacia SMART system. The flow rate was 40 $\mu$ l/min and the mobile phase was 50mM Tris/HCl + 0.15M NaCl, pH 7.2. 80 $\mu$ l fractions were collected and splatted. Activity was detected 15 in fractions corresponding to a retention of 1.16 ml.

Further isolation of the active proteins can be done by SDS PAGE analysis in line with Example II.

Example IV-b carrot root culture

Carrot root cultures were initiated as follows.

- 5 For each individual culture 10 surface sterilised *Daucus*  
carote cv Autumn King seeds were placed into 100 ml MS medium  
containing 25 g/L sucrose and 0.5 g/L MES in sterile 250 ml  
Erlenmyer flasks. Seeds were germinated by shaking at 150 rpm  
in the dark at 25°C for 3 weeks. Leaves and shoots were then  
10 aseptically removed. The roots were replaced into 100 ml  
fresh medium and incubated with shaking for a further 2  
weeks.

- Homogenates were prepared from cold treated and non-cold  
15 treated root cultures as follows. Fast frozen roots were  
ground up 3x in liquid nitrogen in a cold mortar and pestle  
then transferred to a further chilled mortar and pestle and  
ground up with 0.5x volume of ice-cold 50mM Tris.HCl + 10 mM  
EDTA pH 7.4 containing 30 % w/w sucrose. Homogenates were  
20 centrifuged at 10.000 g for 10 minutes at 4°C and the  
supernatant tested for activity as in Example I.  
Significantly more activity was detected in cold treated root  
cultures than in non-cold treated root cultures.

Example V preparation of ice-cream

Root extract from cold acclimatised carrot roots was prepared by scrubbing freshly pulled cold acclimatised (as in example 5 I) carrots in cold water. The tops are removed and the juice extracted employing a domestic juice extractor (Russell Hobbs, model no 9915). The juice was frozen in 1 litre blocks and stored at -20°C prior to collection for use in ice cream trials. The carrot AFP juice was added to the following ice cream formulation:

INGREDIENT	parts by weight
Skimmed Milk Powder	10.000
Sucrose	13.000
MD40	4.000
Locust Bean Gum	0.144
Genulacta L100	0.016
MGP	0.300
Butteroil	8.000
Vanillin	0.012
Water	64.528
Carrot Extract (from cold acclimated carrots containing 1-10 mg AFP per kg)	4.472

Ice-cream was prepared by freezing the above formulation and aeration to 106% overrun.

Measurements were made on fresh sample and on samples which had been abused by storage at  $-10^{\circ}\text{C}$  for a period of 10 days. As a comparison a sample without carrot extract was measured in the same way. The measurements were done as follows:

Samples were equilibrated at  $-18^{\circ}\text{C}$  in a Prolan Environmental cabinet for approximately 12 hours. Three samples were chosen representatively from each batch of ice cream and a slide was prepared from each in a Cryostat temperature control cabinet by smearing a thin layer of ice cream from the centre of each block onto a microscopic slide. A single drop of white spirit was applied to the slide and a cover slip was then applied. Each slide, in turn, was then transferred to a temperature controlled microscope stage (Leit LaborLux S, Leica x10 objective, temperature  $-18^{\circ}\text{C}$ ). Images of ice-crystals (about 400 individual ice-crystals) were collected and relayed through a video camera (Sanyo CCD) to an image storage and analysis system (LEICA Q520MC).

The stored ice crystal images were highlighted manually by drawing around the perimeter which then highlights the whole crystal. Images of the highlighted crystals were then measured using the image analysis software which counts the number of pixels required to complete the longest straight line (length), shortest straight line (breadth), the aspect ratio (length/breadth). The data for each individual ice crystal of a batch of ice cream was imported into a spreadsheet where analysis of the data set was carried out to find the mean, and standard deviation.

The ice Cream Hardness Measurements were carried out using a Hounsfield H10KM Universal Tester, a Hounsfield 100N Load Cell and a 10cm Cylindrical Stainless steel probe. The ice-cream samples were prepared by 16 Hour incubation of 486ml  
5 ice cream blocks in a Prolan Temperature Control Cabinet set at -18 °C.

The ice cream block was removed from Prolan temperature control cabinet and placed the Hounsfield H10KM Universal  
10 Tester. The 10cm cylindrical probe was pushed into the ice cream block at a constant rate of 400mm/min to a depth of 20mm. The maximum force recorded during the compression was used and expressed as the ice cream Hardness. If cracking or brittle fracture of the sample was observed this was  
15 indicated in the right hand column

The following results were obtained

Sample	Ice Crystal Size Parameters				Material Properties	
	Mean Crystal Length / $\mu\text{m}$	Mean Crystal Breadth / $\mu\text{m}$	Mean Crystal Shape Factor / -	Mean Crystal Aspect Ratio / -	Hardness / N	Brittle Fracture observation
Carrot AFP - fresh	26.79 $\pm$ 1.3	19.00 $\pm$ 0.9	1.15 $\pm$ 0.013	1.43 $\pm$ 0.024	40.8	Yes
Carrot AFP - Abused	33.48 $\pm$ 1.3	24.61 $\pm$ 0.9	1.13 $\pm$ 0.013	1.37 $\pm$ 0.020	59.9	Yes
Cont.- Fresh	33.67 $\pm$ 1.1	24.79 $\pm$ 0.8	1.12 $\pm$ 0.008	1.38 $\pm$ 0.018	27.3	No
Cont.- Abused	61.77 $\pm$ 2.7	46.54 $\pm$ 2.0	1.11 $\pm$ 0.010	1.37 $\pm$ 0.020	32.7	No

5 This proves that carrot AFP has good ice recrystallisation inhibition properties.

**Example VI**

The peptide sequences shown in Example III were analyzed as to their suitability for degenerate oligonucleotide primer design. Part of Peptide D (GLY-PRO-VAL-PRO-LEU-PHE-PHE-PRO) was chosen and the primer cp3 (GGI CCI GTI CCI YTI TTY TTY CC, where I= inosine and Y=C or T) was synthesized (Genosys).

10 First strand cDNA was prepared from 5 µg cold acclimated (1 month as in example I) carrot root RNA using Superscript Reverse Transcriptase (Stratagene) and an oligonucleotide primer OG1 (GAGAGAGGATCCTCGAG(T)<sup>15</sup>) according to the manufacturer's instructions. 1% of the first strand cDNA  
15 reaction was used as template, together with cp3 and OG1 primers, in subsequent PCR. The reactions were carried out in a thermal cycler using Taq DNA polymerase (Gibco BRL) for 30 cycles (1 minute at 94 °C, 1 minute at 50 °C and 1 minute at 72 °C) according to the manufacturer's  
20 instructions. All primers were used at a concentration of 1 µM. The resulting ~800 bp PCR product was gel purified and cloned into the pTAG vector (R&D Systems) according to the manufacturer's instructions. The cloned cp3 PCR product was sequenced using the dideoxy sequencing method employed by  
25 the Sequenase kit (USB). The cp3 nucleotide sequence and deduced amino acid sequence were substantially similar to:

GGGCCGGTGCCGCTGTTCTTCCCTCAGCTTACGAACTAACTTGT'TTAGACTTATCGTTT  
1 -----+-----+-----+-----+-----+ 60  
5 a G P V P L F F P Q L T K L T C L D L S F -  
  
AACAACTTTTGGGTGTAATCCCTCCTCAGCTTCCACTCTTCCGAACCTTAAAGCCCTG  
61 -----+-----+-----+-----+-----+ 120  
10 a N K L L G V I P P Q L S T L P N L K A L -  
  
CACTTAGAACGTAACGAACTCACCAGGTGAAATCCCCGATATCTTTGGGAATTTTGCTGGA  
121 -----+-----+-----+-----+-----+ 180  
a H L E R N E L T G E I P D I F G N F A G -  
  
TCCCCGACATATATCTTTCGCATAACCAGCTCACCAGGTTTGT'TCCAAAACCTTTTGCT  
181 -----+-----+-----+-----+-----+ 240  
15 a S P D I Y L S H N Q L T G F V P K T F A -  
  
AGAGCAGATCCAATTAGGCTCGACTTCTCAGGGAACAGACTAGAAGGTGATATTCATTC  
20 241 -----+-----+-----+-----+-----+ 300  
a R A D P I R L D F S G N R L E G D I S F -  
  
TTGTTTGGGCCTAAAAACGCTTGGAAATGCTAGATTTTTCAGGAAACGTGCTTAGTTTC  
25 301 -----+-----+-----+-----+-----+ 360  
a L F G P K K R L E M L D F S G N V L S F -  
  
AATTTCTCCAGGGTGCAGGAGTTTCCACCCTCTTTGACATACTTAGACTTGAACCATAAC  
30 361 -----+-----+-----+-----+-----+ 420  
a N F S R V Q E F P P S L T Y L D L N H N -  
  
CAGATCAGCGGAAGTCTGTGAGTGAATTGGCTAAATTGGACCTGCAGACATTTAACGTC  
35 421 -----+-----+-----+-----+-----+ 480  
a Q I S G S L S S E L A K L D L Q T F N V -  
  
AGTGATAATAATCTCTGCGGCAAGATTCCAACAGGGGAAACCTCCAGAGATTCGACCGT  
40 481 -----+-----+-----+-----+-----+ 540  
a S D N N L C G K I P T G G N L Q R F D R -  
  
ACGGCCTATCTCCACAACAGTTGCTTGTGTGGTGTCTCCATTGCCAGAATGCTAGTTACCA  
45 541 -----+-----+-----+-----+-----+ 600  
a T A Y L H N S C L C G A P L P E C \*  
  
TGCAAAATGTGCCTTAAGGTTATCTTTGTAATGAGATATATTATGCAGCTCAAGGCAGAG  
601 -----+-----+-----+-----+-----+ 660



```

                    CAATAAGTTTTCCTAATTGTTATAGTAAGATATTATTGTATTTCACAGAAAGTGTCTAC
661 -----+-----+-----+-----+-----+-----+ 720
5
                    TAGGATTCGTAATATATTATAATTGCTCATAATTGTATCTGTTAATCTGTAATCCAAAA
721 -----+-----+-----+-----+-----+-----+ 780
                    ACCTTTATGTATTGGTTTGACACTTTTGAGCTTTAAAAAAAAAAAAAAAAAAAA
781 -----+-----+-----+-----+-----+-----+ 829
10

```

Listing II

In order to obtain the full coding region for the carrot  
 15 AFP, a cDNA library was constructed. A poly (A)+ quick  
 column (Stratagene) was used to purify mRNA from 500 µg CA  
 (1 month) carrot total RNA, according to manufacturer's  
 instructions. All resulting poly (A)+ RNA was used for cDNA  
 synthesis and subsequent library construction using the  
 20 lambda ZAP vector kit (Stratagene).  $1 \times 10^5$  recombinant  
 phage clones were screened by hybridization using the cp3  
 PCR product as a  $^{32}\text{P}$  labelled probe.

Positive plaques were screened to purity and phage-mids  
 25 excised before the inserts were characterised by DNA  
 sequence analysis. Two cDNA clones were sequenced to  
 completion. Although the 5' and 3' untranslated regions  
 contained some sequence variability, the coding regions  
 were identical. The coding regions of the two cDNA clones  
 30 were substantially similar to:

5 a 13 ATGAATATTGAATCATCTTTCTGCCCTATTTTGTGCATATGCATGATTTTCCTCTGCCTT 72  
M N I E S S F C P I L C I C M I F L C L -  
a 73 CCAAACCTCTCTGCATCACAAAGATGCAACAACGACAAGCAAGCTTTACTCCAAATC 132  
P N L S A S Q R C N N N D K Q A L L Q I -  
10 a 133 AAAACAGCCTTGAAAAACCCACCATTACAGACTCATGGGTGTCAGACGACGATTGTGT 192  
K T A L K N P T I T D S W V S D D D C C -  
15 a 193 GGTGGGACCTAGTCGAATGTGACGAAACCAGCAACCGCATAATTTCCCTCATAATTCAA 252  
G W D L V E C D E T S N R I I S L I I Q -  
20 a 253 GACGACGAAGCTCTCACCGGCCAAATCCACCTCAGGTGGGAGACCTACCATACCTCCAA 312  
D D E A L T G Q I P P Q V G D L P Y L Q -  
25 a 313 GCCTTATGGTTCGTAAATCCCAATCTTTTCGGAAAAATCCCAGAAGAAATTTCTGCA 372  
A L W F R K L P N L F G K I P E E I S A -  
a 373 CTCAAAGACCTAAATCCCTCAGACTCAGCTCGACCAGTCTCAGTGGCCCTGTCCCTTTA 432  
L K D L K S L R L S S T S L S G P V P L -  
30 a 433 TTCTCCCTCAGCTTACGAACTAAGTTGTTTAGACTTATCGTTTAACTTTTGGGT 492  
F F P Q L T K L T C L D L S F N K L L G -  
35 a 493 GTAATCCCTCCTCAGCTTTCCACTCTTCCGAACCTTAAAGCCCTGCACTTAGAACGTAAC 552  
V I P P Q L S T L P N L K A L H L E R N -  
40 a 553 GAACCTCACCGGTGAAATCCCGATATCTTTGGGAATTTGCTGGATCCCGGACATATAT 612  
E L T G E I P D I F G N F A G S P D I Y -  
45 a 613 CTTTCGCATAACCAGCTCACCGGGTTTGTTCACAACTTTTGCTAGAGCAGATCCAATT 672  
L S H N Q L T G F V P K T F A R A D P I -  
50 a 673 AGGCTCGACTTCTCAGGGAACAGACTAGAAGGTGATATTTTCACTTGTGTTGGGCCTAAA 732  
R L D F S G N R L E G D I S F L F G P K -  
a 733 AAACGCTTGGAATGCTAGATTTTTCAGGAAACGTGCTTAGTTTCAATTTCTCCAGGGTG 792  
K R L E M L D F S G N V L S F N F S R V -  
55 a 793 CAGGAGTTTCCACCCTCTTTGACATACTTAGACTTGAACCATAACCAGATCAGCGGAAGT 852  
Q E F P P S L T Y L D L N H N Q I S G S -  
60 a 853 CTGTCGAGTGAATTGGCTAAATTGGACCTGCAGACATTTAACGTGATGATAATAATCTC 912  
L S S E L A K L D L Q T F N V S D N N L -  
65 a 913 TCGGGCAAGATTCCAACAGGGGAAACCTCCAGAGATTCGACCGTACGGCCTATCTCCAC 972  
C G K I P T G G N L Q R F D R T A Y L H -  
a 973 AACAGTTGCTTGTGTGGTGTCCATTGCCAGAATGCTAG 1011  
N S C L C G A P L P E C \* -  
70

Listing I

Partial sequence analysis of 4 other clones also indicated that they contained the same coding region as the fully sequenced clones and thus all the positives from the  
5 library screen were likely to represent transcripts from the same gene. The existence of only one copy of the AFP gene in the carrot genome was further substantiated by the fact that Southern analysis of restriction enzyme digested carrot genomic DNA suggested that only one fragment  
10 hybridized to the probe.

### Example VII

In order to prove that the carrot cDNA as shown in example  
5 VI represented an AFP, expression of the coding region was  
carried out as follows. One of the cDNAs was first cloned  
into an intermediate pUC plasmid vector (Messing, 1983)  
containing a double CaMV 35S promoter (Guerineau, J. F.,  
Woolsten, S., Brooks, L., and Mollineaux, P. (1988))  
10 expression cassette, and then into a binary vector, as  
described below. All enzymes used were supplied by Gibco  
BRL and used according to the manufacturer's instructions.

The pBluescript phagemid (Stratagene) containing the cDNA  
15 clone was digested with *Xho* I and the recessed 3' termini  
filled in using the Klenow fragment of DNA polymerase I.  
The cDNA fragment was then released from the vector by  
digestion with *Eco* RI. The *Eco* RI/blunt cDNA fragment was  
then cloned into the *Eco* RI/blunt digested intermediate pUC  
20 plasmid vector. The CaMV 35S-cDNA expression cassette was  
then subcloned as a partial *Hind* III fragment into  
*Hind* III-cut pBin 19 binary vector (Bevan 1984). The binary  
vector construct was then introduced into tobacco using  
*Agrobacterium* mediated transformation (as described in  
25 Draper, J., Scott, R., Armatage, P., and Walden, R.  
(1988)).

Transgenic tobacco callus was analyzed for expression of  
recrystallisation inhibition activity as soon as sufficient  
30 kanamycin resistant material was regenerated. Small scale  
protein extracts were made from several independent

kanamycin resistant calli plus some wild type tobacco callus. Approximately 2 g tissue was ground up in 1-2 mls sucrose buffer (30 % sucrose 50 mM Tris, 10 mM EDTA, 20 mM ascorbate, pH 7.2) using a mortar and pestle. 5 The solution was centrifuged at 10,000 xg for 2 minutes and the supernatant removed to a fresh tube. An aliquot of 3 µl of protein extract was tested for recrystallisation activity using the sucrose sandwich splat assay method of example I. All kanamycin resistant callus extracts tested 10 demonstrated recrystallisation inhibition activity.

Stable transgenic tobacco plants expressing the carrot AFP have also been produced. Leaf extracts from wild-type and transgenic tobacco plants have been subjected to northern 15 analysis using the AFP cDNA as a probe. The AFP message was only detectable in the transgenic tobacco plants. This suggests that the AFP message is stable in the greenhouse grown transgenic tobacco plants. When compared with the native carrot transcript, the tobacco AFP transcript 20 appears to be slightly bigger. This discrepancy can be explained by the method of construction of the AFP expression cassette. Because the CaMV 35S polyadenylation signal is most 3' in the construct, it is likely that this signal is used in the transgenic AFP message, giving rise 25 to a longer transcript. Leaf extracts from wild-type and transgenic tobacco plants have also been analyzed by western blotting using a carrot AFP antibody. A cross-reacting protein was only detected in the transgenic tobacco plants. Despite the difference in transcript size, 30 the protein produced in tobacco appears to be the same size as the native carrot AFP.

The above data provides the proof that the protein purified from carrot and the corresponding cDNA represent an active AFP.

**CLAIMS**

1. Antifreeze polypeptides which can be obtained from carrots and which have an apparent molecular weight on SDS-PAGE of 36 kDa and isoforms or derivatives thereof.
2. Antifreeze polypeptides comprising one or more fragments (A-E) of the amino acid sequence as follows:
  - (A) LEU-PRO-ASN-LEU-PHE-GLY-LYS
  - (B) ILE-PRO-GLU-GLU-ILE-SER-ALA-LEU-LYS
  - (C) LEU-THR-X-LEU-ASP-LEU-SER-PHE-ASN-LYS
  - (D) SER-LEU-ARG-LEU-SER-SER-THR-SER-LEU-SER-GLY-PRO-VAL-PRO-LEU-PHE-PHE-PRO-GLN-LEU-X-LYS
  - (E) X-X-GLY-VAL-ILE-PRO-X-GLN-LEU-SER-THR-LEU-PRO-ASN-LEU-LYSand isoforms or derivatives thereof
3. Antifreeze polypeptides comprising the fragments (A-E) of claim 2.
4. Antifreeze polypeptides having an amino acid sequence as represented in Listing 1 and isoforms and derivatives thereof.

5. An isolated nucleic acid sequence encoding the antifreeze polypeptide of one or more of claims 1-4 and alleles thereof.
6. An isolated nucleic acid sequence substantially corresponding to the gene sequence of Listing I and alleles thereof.
7. Method of obtaining polypeptides according to one or more of claims 1-4 whereby the polypeptide is isolated from cold-acclimatised carrots.
8. Method of obtaining polypeptides according to one or more of claims 1-4, whereby the polypeptide is expressed by a genetically modified organism.
9. Method according to claim 8, whereby the organism is a microorganism, a plant or a cell culture.
10. An antibody capable of specifically binding the polypeptide of claim 1, 2, 3 or 4.
11. A polypeptide that is immunologically related to the polypeptide of claim 1, 2, 3 or 4 as determined by its cross reactivity with an antibody of claim 10.
12. Food product comprising a polypeptide of claim 1, 2, 3, 4 or 11 with the proviso that the food product is not a carrot containing the polypeptide at naturally occurring levels.



13. Food product of claim 12 being a frozen confectionery product or a frozen vegetable.
14. Method of producing a food product comprising an antifreeze polypeptide according to one or more claims 1, 2, 3 or 4, comprising the steps of
  - (a) adding to the food product a composition comprising said antifreeze polypeptide; or
  - (b) in situ production of said antifreeze polypeptide.
15. Use of the polypeptide of claims 1, 2, 3 or 4 for increasing the frost tolerance of plants.
16. Microorganisms, cell line or plant capable of expressing the polypeptide of claims 1, 2, 3 or 4, with the proviso that the plant is not an unmodified carrot plant.

# PATENT COOPERATION TREATY

## PCT

### INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference <b>F 7371 (V)</b>	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. <b>PCT/EP97/06181</b>	International filing date (day/month/year) <b>06/11/1997</b>	Priority date (day/month/year) <b>19/11/1996</b>
International Patent Classification (IPC) or national classification and IPC <b>C12N15/29</b>		
Applicant <b>UNILEVER N.V. et al.</b>		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 5 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand  <b>19/05/1998</b>	Date of completion of this report  <b>0 6. 04. 99</b>
Name and mailing address of the international preliminary examining authority:   <b>European Patent Office D-80298 Munich Tel. (+49-89) 2399-0 Tx: 523656 epmu d Fax: (+49-89) 2399-4465</b>	Authorized officer  <b>Merlos-Lange, A.M.</b>  Telephone No. (+49-89) 2399 8559  

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP97/06181

## I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

### Description, pages:

1-32 as originally filed

### Claims, No.:

1-16 as received on 29/12/1998 with letter of 21/12/1998

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:  
☐ the claims, Nos.:  
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

## V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

### 1. Statement

Novelty (N)	Yes:	Claims 2, 3, 4, 5, 6, 7, 8, 9
	No:	Claims 1, 10-16
Inventive step (IS)	Yes:	Claims 2-4
	No:	Claims 1, 5-16
Industrial applicability (IA)	Yes:	Claims 1-16
	No:	Claims

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/EP97/06181

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**2. Citations and explanations**

**see separate sheet**

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

**s e separate sheet**

- 1). The amended set of claims 1-16 appears to be in conformity with the requirements of Art. 34(2,b) PCT.
- 2). Document WO 98/04148 claims a priority of 26.07.1996 and may therefore become relevant for the assessment of novelty when the present application enters the european phase.
- 3). With respect to document WO 92/22581, the subject-matter of claims 1 and 10 to 16 does not appear to be novel and inventive according to Art. 33(2), (3) PCT. Cited document discloses the isolation of a number of antifreeze polypeptides derived from intracellular spaces of plant cells, one of them having a molecular weight of 36 kDa and the amino acid sequence shown in TABLE 1 (see also page 23). The polypeptides are described to may prove useful for increasing survival and productivity in overwintering crops and in production of frozen foods and cryogenic storage of biological tissue (see page 30 and claims). The production of antibodies for different purposes is also disclosed.  
Claim 1 as amended refers to a polypeptide having antifreeze activity defined by its apparent molecular weight on SDS-PAGE of 36 kD as well as isoforms or derivatives still possessing antifreeze activity.  
In the absence of additional technical features, the polypeptide is not sufficiently precise defined so as to be clearly distinguishable from that (those) disclosed in the above cited document. The molecular weight is not a reliable feature in particular when it is the single one given to define the claimed protein (see also page 3 of the application which mentions the variations in the determination of molecular weight and which defines the term "apparant molecular weight of 36 kDa" as embracing variations from 30 to 40 kDa or from 34 to 38 kDa). With respect to cited document, there is further no reason to doubt that the disclosed antifreezing polypeptides e.g. those having a molecular weight of 36 and 30 kDa respectively do not exhibit antifreezing properties. This is also true for the isoforms and derivatives which may even not be different from the other polypeptides disclosed in WO 92/22581.  
Therefore, claim 1 as well as dependent claims 10-16 are not considered to be in conformity with the requirements of Art. 33(2), (3) PCT.
- 4). With respect to the above cited document, dependent claims 5 and 7-9 which lack

any particular features do not appear to involve an inventive step according to Art. 33(3) PCT. The identification and isolation of the corresponding nucleic acid and the different methods of obtaining antifreeze polypeptides according to claim 1 appear obvious and do not require particular inventive skill.

- 5). The expression "... capable of expressing the polypeptide of ..." used to define the microorganisms, cell line or plant of claim 16, has no limiting effect. Any host may express the polypeptides, i.e. is capable of expressing them, if it is transformed with the appropriate expression plasmid. At present, claim 16 is not clearly directed to such transformed host cells or plants (Art. 6 PCT).

## PATENT COOPERATION TREATY

## PCT

REC'D 08 APR 1999

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## 5650 INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference F 7371 (V)	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/EP97/06181	International filing date (day/month/year) 06/11/1997	Priority date (day/month/year) 19/11/1996
International Patent Classification (IPC) or national classification and IPC C12N15/29		
Applicant UNILEVER N.V. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 5 sheets, including this cover sheet.

- ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 19/05/1998	Date of completion of this report 06.04.99
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. (+49-89) 2399-0 Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer Merlos-Lange, A.M. Telephone No. (+49-89) 2399 8559 

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/EP97/06181

**I. Basis of the report**

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

**Description, pages:**

1-32 as originally filed

**Claims, No.:**

1-16 as received on 29/12/1998 with letter of 21/12/1998

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:  
☐ the claims, Nos.:  
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

Novelty (N)	Yes:	Claims	2, 3, 4, 5, 6, 7, 8, 9
	No:	Claims	1, 10-16
Inventive step (IS)	Yes:	Claims	2-4
	No:	Claims	1, 5-16
Industrial applicability (IA)	Yes:	Claims	1-16
	No:	Claims	



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/EP97/06181

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2. Citations and explanations

**see separate sheet**

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

**see separate sheet**

- 1). The amended set of claims 1-16 appears to be in conformity with the requirements of Art. 34(2,b) PCT.
- 2). Document WO 98/04148 claims a priority of 26.07.1996 and may therefore become relevant for the assessment of novelty when the present application enters the european phase.
- 3). With respect to document WO 92/22581, the subject-matter of claims 1 and 10 to 16 does not appear to be novel and inventive according to Art. 33(2), (3) PCT. Cited document discloses the isolation of a number of antifreeze polypeptides derived from intracellular spaces of plant cells, one of them having a molecular weight of 36 kDa and the amino acid sequence shown in TABLE 1 (see also page 23). The polypeptides are described to may prove useful for increasing survival and productivity in overwintering crops and in production of frozen foods and cryogenic storage of biological tissue (see page 30 and claims). The production of antibodies for different purposes is also disclosed.  
Claim 1 as amended refers to a polypeptide having antifreeze activity defined by its apparent molecular weight on SDS-PAGE of 36 kD as well as isoforms or derivatives still possessing antifreeze activity.  
In the absence of additional technical features, the polypeptide is not sufficiently precise defined so as to be clearly distinguishable from that (those) disclosed in the above cited document. The molecular weight is not a reliable feature in particular when it is the single one given to define the claimed protein (see also page 3 of the application which mentions the variations in the determination of molecular weight and which defines the term "apparant molecular weight of 36 kDa" as embracing variations from 30 to 40 kDa or from 34 to 38 kDa). With respect to cited document, there is further no reason to doubt that the disclosed antifreezing polypeptides e.g. those having a molecular weight of 36 and 30 kDa respectively do not exhibit antifreezing properties. This is also true for the isoforms and derivatives which may even not be different from the other polypeptides disclosed in WO 92/22581.  
Therefore, claim 1 as well as dependent claims 10-16 are not considered to be in conformity with the requirements of Art. 33(2), (3) PCT.
- 4). With respect to the above cited document, dependent claims 5 and 7-9 which lack

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/EP97/06181

any particular features do not appear to involve an inventive step according to Art. 33(3) PCT. The identification and isolation of the corresponding nucleic acid and the different methods of obtaining antifreeze polypeptides according to claim 1 appear obvious and do not require particular inventive skill.

- 5). The expression "... capable of expressing the polypeptide of ..." used to define the microorganisms, cell line or plant of claim 16, has no limiting effect. Any host may express the polypeptides, i.e. is capable of expressing them, if it is transformed with the appropriate expression plasmid. At present, claim 16 is not clearly directed to such transformed host cells or plants (Art. 6 PCT).

## ANNEX

## CLAIMS

1. Polypeptides having antifreeze activity which can be obtained from carrots and which have an apparent molecular weight on SDS-PAGE of 36 kDa and isoforms or derivatives thereof which still possess antifreeze activity.
2. Polypeptides having antifreeze activity comprising one or more fragments (A-E) of the amino acid sequence as follows:

(A) LEU-PRO-ASN-LEU-PHE-GLY-LYS

(B) ILE-PRO-GLU-GLU-ILE-SER-ALA-LEU-LYS

(C) LEU-THR-X-LEU-ASP-LEU-SER-PHE-ASN-LYS

(D) SER-LEU-ARG-LEU-SER-SER-THR-SER-LEU-SER-GLY-PRO-  
VAL-PRO-LEU-PHE-PHE-PRO-GLN-LEU-X-LYS

(E) X-X-GLY-VAL-ILE-PRO-X-GLN-LEU-SER-THR-LEU-PRO-  
ASN-LEU-LYS

and isoforms or derivatives thereof which still possess antifreeze activity.

AMENDED SHEET

3. Polypeptides having antifreeze activity comprising the fragments (A-E) of claim 2.
4. Polypeptides having antifreeze activity having an amino acid sequence as represented in Listing 1 and isoforms and derivatives thereof which still possess antifreeze activity.
5. An isolated nucleic acid sequence encoding the antifreeze polypeptide of one or more of claims 1-4 and alleles thereof encoding polypeptides which still possess antifreeze activity.
6. An isolated nucleic acid sequence corresponding to the gene sequence of Listing I and alleles thereof encoding polypeptides which still possess antifreeze activity .
7. Method of obtaining polypeptides according to one or more of claims 1-4 whereby the polypeptide is isolated from cold-acclimatised carrots.
8. Method of obtaining polypeptides according to one or more of claims 1-4, whereby the polypeptide is expressed by a genetically modified organism.
9. Method according to claim 8, whereby the organism is a micro-organism, a plant or a cell culture.

AMENDED SHEET

10. An antibody capable of specifically binding the polypeptide of claim 1, 2, 3 or 4.
11. A polypeptide which has antifreeze activity that is immunologically related to the polypeptide of claim 1, 2, 3 or 4 as determined by its cross reactivity with an antibody of claim 10.
12. Food product comprising a polypeptide of claim 1, 2, 3, 4 or 11 with the proviso that the food product is not a carrot.
13. Food product of claim 12 being a frozen confectionery product or a frozen vegetable.
14. Method of producing a food product comprising an antifreeze polypeptide according to one or more claims 1, 2, 3 or 4, comprising the steps of
  - (a) adding to the food product a composition comprising said antifreeze polypeptide; or
  - (b) in situ production of said antifreeze polypeptide.
15. Use of the polypeptide of claims 1, 2, 3 or 4 for increasing the frost tolerance of plants.

AMENDED SHEET

16. Micro-organisms, cell line or plant capable of expressing the polypeptide of claims 1, 2, 3 or 4, with the proviso that the plant is not an unmodified carrot plant.

AMENDED SHEET

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>F 7371 (V)</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/EP 97/06181</b>	International filing date (day/month/year) <b>06/11/1997</b>	(Earliest) Priority Date (day/month/year) <b>19/11/1996</b>
Applicant <b>UNILEVER N.V. et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. ☐ Certain claims were found unsearchable (see Box I).
2. ☐ Unity of invention is lacking (see Box II).
3. ☒ The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing

☐ filed with the international application.

☒ furnished by the applicant separately from the international application,

☐ but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.

☐ Transcribed by this Authority

4. With regard to the title, ☐ the text is approved as submitted by the applicant  
☒ the text has been established by this Authority to read as follows:

**CARROT ANTIFREEZE POLYPEPTIDES**

5. With regard to the abstract,

☒ the text is approved as submitted by the applicant

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is:

Figure No.            ☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 97/06181

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/29 C07K14/415 C12P21/02 C12N1/21 C07K16/16  
C12N5/04 A23L3/375 A23G9/02 A23G9/04 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K C12P A23L A23G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DUMAN J G ET AL: "THERMAL HYSTERESIS PROTEIN ACTIVITY IN BACTERIA FUNGI AND PHYLOGENETICALLY DIVERSE PLANTS." CRYOBIOLOGY 30 (3). 1993. 322-328., XP000673724 see the whole document ---	1,2
A	WO 92 22581 A (UNIV WATERLOO) 23 December 1992 cited in the application see page 14; claims 20-26 ---	1-4,7, 10-15
A	WO 94 03617 A (UNILEVER PLC ; UNILEVER NV (NL); TOSCHKA HOLGER YORK (NL); VERBAKEL) 17 February 1994 see the whole document ---	5,6,8,9, 16
	--- -/--	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## ° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&amp;" document member of the same patent family

Date of the actual completion of the international search

28 May 1998

Date of mailing of the international search report

04/06/1998

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Maddox, A

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 97/06181

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 11586 A (HSC RES DEV LP ; SEABRIGHT CORP LTD (CA)) 25 April 1996 cited in the application see the whole document ----	5,6,8,9, 12,13,16
A	WO 90 13571 A (DNA PLANT TECHN CORP) 15 November 1990 cited in the application see page 20 - page 22 see page 43 - page 63 ----	12,13,15
A	WO 94 17186 A (UNIV FLORIDA) 4 August 1994 see the whole document ----	15
A	WO 91 08292 A (KEMIRA OY) 13 June 1991 see page 7 ----	15
A	LEE, J., ET AL.: "The reduction of the freezing point of tobacco plants transformed with the gene encoding for the antifreeze protein from winter flounder" J.CELL.BIOCHEM.SUPPL., vol. 14E, 1990, page 303 XP002030248 * abstract R228 * ----	15
E	WO 98 04148 A (UNILEVER PLC ; UNILEVER NV (NL)) 5 February 1998 see the whole document -----	1,2,7, 10-14

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 97/06181

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9222581	A	23-12-1992	AU 1907192	A	12-01-1993
			CA 2110510	A	23-12-1992
			EP 0589928	A	06-04-1994
WO 9403617	A	17-02-1994	AU 4571993	A	03-03-1994
WO 9611586	A	25-04-1996	US 5676985	A	14-10-1997
			AU 3602395	A	06-05-1996
			CA 2202373	A	25-04-1996
			EP 0785727	A	30-07-1997
WO 9013571	A	15-11-1990	US 5118792	A	02-06-1992
			AU 648028	B	14-04-1994
			AU 5814390	A	29-11-1990
			CA 2056434	A	11-11-1990
			EP 0472662	A	04-03-1992
			JP 4505329	T	17-09-1992
WO 9417186	A	04-08-1994	AU 6229894	A	15-08-1994
WO 9108292	A	13-06-1991	FI 895614	A	24-05-1991
			CA 2044606	A	24-05-1991
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			JP 4503013	T	04-06-1992
WO 9804148	A	05-02-1998	EP 0843010	A	20-05-1998
			AU 3443797	A	20-02-1998
			AU 3621297	A	20-02-1998
			AU 3621397	A	20-02-1998
			AU 3693497	A	20-02-1998
			DE 19732135	A	26-02-1998
			DE 19732136	A	29-01-1998
			WO 9804699	A	05-02-1998
			WO 9804146	A	05-02-1998
			WO 9804147	A	05-02-1998
			FR 2751657	A	30-01-1998
			FR 2751513	A	30-01-1998
			GB 2315752	A	11-02-1998
			GB 2315753	A	11-02-1998
			DE 19732132	A	29-01-1998

**Information on patent family members**

PCT/EP 97/06181

Form PCT/ISA/210 (patent family annex) (July 1992)